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# Accepted Manuscript

Complement C3 exacerbates imiquimod-induced skin inflammation and psoriasiform dermatitis

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**Title: Complement C3 exacerbates imiquimod-induced skin inflammation and psoriasiform dermatitis**

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**To the Editor**

The complement system is pivotal in protection against pathogens, but also plays important roles in bridging innate and adaptive immune responses (Scott and Botto, 2015) and in modulating local and systemic inflammation (Markiewski and Lambris, 2007). Activation of complement occurs through three different pathways (classical, alternative and lectin), converges at C3 cleavage and culminates in the formation of the membrane attack complex. The anaphylotoxic fragments, C3a and C5a, generated during the proteolytic cascade, recruit immune cells that can promote the removal of debris and pathogens, but can also cause tissue damage (Markiewski and Lambris, 2007).

The main source of complement is the liver. However, locally produced complement, particularly C3, can modulate inflammation in a variety of organs. There is also evidence that not only immune cells such as macrophages and dendritic cells produce complement components, but also non-immune cells can contribute to local complement synthesis. In the skin keratinocytes are a potential source of C3 (Pasch et al., 2000). A role for C3 in the skin has been identified in UV-induced immune tolerance (Hammerberg et al., 1998) as well as in the protection against T cell mediated inflammation (Purwar et al., 2011). Furthermore, locally produced C3 contributes to the inflammatory responses accompanying wound healing (Rafail et al., 2014), an effect mediated mainly through C5a/C5aR interactions. The latter also play an important role in influencing the skin microbiome (Chehoud et al., 2013). In keeping with these observations in animal models, complement has been shown to have a role in the pathogenesis of many human skin diseases, including psoriasis (Kotnik V., 2011).

Psoriasis is an inflammatory skin disease characterised by epidermal hyperplasia, infiltration of immune cells and secretion of inflammatory cytokines (Tortola et al., 2012). The anaphylotoxic fragments, C3a and C5a, have been found in corneal scale extracts from psoriatic lesions (Takematsu et al., 1986). The C5a from these lesions has been shown to chemoattract monocyte-derived dendritic cells (Mrowietz et al., 2001), indicating that complement may contribute to the inflammatory process in this disease. In the inducible Activator Protein-1-dependent psoriasis-like mouse model the S100A8-S100A9 complex that promotes skin inflammation has been shown to upregulate C3 expression (Schonthaler et al., 2013). Psoriasis-like dermatitis can be induced by topical application of the toll-like receptor 7 (TLR7) agonist imiquimod (IMQ) (Van der Fits et al., 2009) in the form of Aldara® cream (Walter et al., 2013). Here we explore the role of complement in IMQ-mediated psoriasiform dermatitis.

We first tested whether cutaneous IMQ treatment induces local C3 synthesis (experimental methods are provided as Supplementary information online) and found a progressive increase in C3 mRNA in the skin with repeated IMQ applications (Figure 1a and Figure S1a). Immunohistochemistry demonstrated that the C3 expression was predominantly in the dermis (Figure S1b). *In vitro* experiments with isolated dermal stromal cells (DSCs) showed that these cells can produce C3 upon stimulation with inflammatory cytokines known to be induced in IMQ-treated skin, but not upon direct challenge with a TLR7 agonist (Figure S1c). To test whether C3 contributes to the psoriatic-like lesions induced by IMQ, we then treated WT and  $C3^{-/-}$  mice for 7 consecutive days. The treatment resulted in skin thickening, scaling and erythema (Figure 1b). However, mice lacking C3 displayed less skin inflammation with significantly reduced ear thickness and lower scores for erythema and scaling compared to WT mice (Figure 1c). Consistent with the reduced skin response in  $C3^{-/-}$  mice, we found

significantly fewer infiltrating neutrophils, but slightly more monocytes, and no difference in the number of resident  $\gamma\delta$  T cells compared to WT animals (Figure 1d). IL-17 secretion by  $\gamma\delta$  T cells plays a key role in the IMQ-induced psoriasis model (van der Fits et al., 2009) and our data confirmed that IL-17 secretion was mainly restricted to these cells. Importantly, the frequency of IL-17-positive  $\gamma\delta$  T cells in both skin and draining LN was significantly lower in the absence of C3 (Figure 1e and 1f). All animals were handled in accordance with the institutional guidelines and the UK Home Office approved the procedures.

We next evaluated whether C3 contributes to the local inflammatory response. To this end we analysed the gene expression of a selected number of cytokines/chemokines known to be induced in the skin by IMQ treatment (Di Meglio et al., 2014; Walter et al., 2013). At the peak of clinical inflammation on day 7, the  $C3^{-/-}$  mice did not show any obvious differences in inflammatory gene-expression (Figure 2a). However, at the onset of the clinical pathology, day 3, when the cytokine/chemokine gene response peaks (Di Meglio et al., 2014),  $C3^{-/-}$  mice had a markedly reduced response, suggesting that C3 modulates the inflammatory gene induction that precedes the clinical manifestations. To substantiate this we carried out a time-course analysis of the skin gene expression after 3 days of IMQ treatment. This showed that in the absence of C3 the resolution of the IMQ-triggered inflammation was faster compared to WT mice. Twenty-four hours after the last application,  $C3^{-/-}$  mice had significantly reduced levels of all genes analysed, namely IL-1 $\alpha$ , TNF- $\alpha$ , IL-17a, IL-23a, CXCL1 and CCL2 (Figure S2a). Consistent with this, thickening of the skin assessed by histology was significantly reduced in the  $C3^{-/-}$  mice (Figure 2b-c). Interestingly, the altered skin pathology in the IMQ-treated  $C3^{-/-}$  mice appeared to affect mainly the epidermis. Untreated skin of WT and  $C3^{-/-}$  mice was histologically indistinguishable.

In summary, we demonstrate that C3 is involved in the development and resolution of the psoriasiform skin inflammation induced by short-term treatment with IMQ. The pro-inflammatory effect of C3 is likely to be mediated by several mechanisms. In the absence of C3 the expression of psoriasis-relevant genes in the skin was impaired, neutrophil infiltration into the inflamed site was decreased and IL-17 production by  $\gamma\delta$  T cells in the skin and the draining LNs was reduced. Taken together these data support a pro-inflammatory role of C3 during psoriasis-like skin inflammation.

## CONFLICT OF INTEREST

The authors declare no conflict of interest

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## FIGURE LEGENDS

### Figure 1. IMQ-induced skin inflammation and psoriasiform dermatitis is impaired in C3-deficient mice.

IMQ was applied for 7 days to the ear of BALB/c WT (black square) and BALB/c.C3<sup>-/-</sup> (red circle) mice. (a) C3 expression in whole skin quantified by qRT-PCR 24 hrs after the last application (n=4). Naïve=untreated mice, mean ± SEM relative to cyclophilin, unpaired *t* test. (b) Representative pictures of IMQ-treated ears at day 7. (c) Ear thickness (mean ± SEM) and clinical scores (mean ± SD) for erythema and scaling. Representative experiment of two, Mann-Whitney test. (d) Number of CD45<sup>+</sup> cells, neutrophils (Ly-6G<sup>+</sup>), monocytes (CD11b<sup>+</sup>Ly-6C<sup>+</sup>Ly-6G<sup>-</sup>) and γδ T cells (CD4<sup>-</sup>CD3<sup>+</sup>γδ TCR<sup>+</sup>) at day 7. (e-f) Proportion of IL-17<sup>+</sup> γδ T cells from skin (e) and LN (f) following *in vitro* restimulation. Mean ± SEM, unpaired *t* test, \**p* <0.05, \*\**p* <0.01, \*\*\**p* <0.01.

### Figure 2. Reduced early pro-inflammatory response and skin thickening in IMQ-treated C3-deficient mice.

(a) IMQ was applied for 3 or 7 days to the ear of BALB/c WT (black square) and BALB/c.C3<sup>-/-</sup> (red circle) mice. Gene expression was analysed in whole skin 24 hrs after the last IMQ exposure (n=3-5/group). Naïve=untreated mice, mean ± SEM relative to the control gene cyclophilin (SYBR Green assay) or GADPH (Taqman assay) levels. (b) Representative images showing H&E staining of cross-sectional ear skin from BALB/c and BALB/c.C3<sup>-/-</sup> mice untreated and 48 hrs after the 3<sup>rd</sup> IMQ application. The epidermis/dermis boundaries are indicated. Scale bars represent 50 μM. (c) Quantification of whole skin and epidermal thickness calculated using Image J software. Each symbol represents an individual mouse (n=4). Mean ± SEM; unpaired *t* test; ns=non-significant; \**p* <0.05, \*\**p* <0.01



